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13. Abstract (Maximum 200 Words) We previously identified the psoriasin (S100A7) gene as being differentially expressed between different stages of human breast cancer. High psoriasin expression was seen in DCIS compared to normal and invasive breast tissue. Protein expression was also found to be present in both the nucleus and the cytoplasm. Currently, there are no data on the biological role of psoriasin, and how its altered expression contributes to the biological process of invasion. To identify potential pathways or cellular processes in which psoriasin participates, we have utilized the yeast two-hybrid assay to identify interacting proteins. Full-length psoriasin was used to screen 17.3×10^6 clones from a normal human mammary cDNA library. Two centrosomal proteins, ranBPM and hGCP3, were determined to be true positives in the yeast assay and selected for further study. Co-immunoprecipitation studies confirmed the interaction between psoriasin and ranBPM. As well, confocal microscopy analyses show that psoriasin does indeed localize to the centrosome in two different human breast cancer cell lines that have been stably transfected with the psoriasin gene. Quantitative RT-PCR of selected human breast tumors was performed to determine the level of psoriasin and ranBPM expression in relation to clinicopathological parameters. The biological importance of the psoriasin-ranBPM interaction is under investigation				
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	9
Reportable Outcomes.....	10
Conclusions.....	11
References.....	12
Appendices.....	13

“The Role of the S100a7/ranBPM interaction in Human Breast Cells”

Introduction

The discovery of genes showing altered expression levels at different stages of breast cancer progression has many immediate uses: they can serve as bio-markers to predict patient outcome, determination of a treatment regime, as well as new potential targets for drug development. The need for such markers is demonstrated by the observation of an increasing number of patients each year with pre-invasive disease¹. Utilizing the resources within the NCIC-Manitoba Breast Tumor Bank, we have shown the psoriasin (S100A7) gene to be a candidate bio-marker². The contribution of psoriasin in the transition from pre-invasive to invasive disease has not been determined. We have begun to determine a biological function for psoriasin by demonstrating that it specifically interacts with the centrosomal protein ranBPM (for summary of ranBPM see ref 3), as well as colocalizing to the centrosome in human breast cancer cell lines. In recent years there has been an explosion of work done on the centrosome and it's potential link to cancer development through and progression^{4,5,6}. The aim of this work is to examine the importance of the psoriasin/ranBPM interaction in terms of promoting specific aspects of breast cancer progression such as adhesion, motility and invasiveness.

BODY OF REPORT

The accomplishments over the first year of this award are detailed below in the context of the three major tasks defined in the statement of work

TECHNICAL OBJECTIVE / AIM 1

1. 1-6, construct ranBPM and S100A7 (psoriasin) epitope tagged expression constructs
2. 6-12, Conduct transient transfection assay with ranBPM and S100A7 to co-localize using confocal microscopy and study biochemical interaction by immunoprecipitation

Aim 1.1

- ◆ Psoriasin protein encoding cDNA sequence cloned into pShooter (InVitrogen) to produce a C-terminal myc-epitope tagged fusion protein. The fidelity of insert confirmed by DNA sequencing. High expression was observed in cells that do not express psoriasin at the Western Blot level. Psoriasin was also cloned into pcDNA3.1 (InVitrogen) to produce a untagged protein product. Vector has T7 promoter: can generate and detect a protein product by invitro transcription-translation kits.
- ◆ RanBPM cloned into pFLAG (Sigma) to produce a C-terminal FLAG epitope tagged fusion protein. Fidelity of insert confirmed by DNA sequencing. The construct was transfected into several cell lines under transient conditions to determine if fusion protein could be detected by Western Blot. All attempts failed. We assumed that there was something wrong with the vector so RanBPM was then re-cloned into pcDCA4 HisMax (InVitrogen) to produce an N-terminal His-Xpress epitope tagged fusion protein. This vector has an enhancer site in the promoter to produce more mRNA than by the CMV promoter alone. pcDNA4 also has T7 promoter: can generate and detect a protein product by invitro transcription-translation kits. I was

able to detect the fusion protein at the correct size by Western blot, but expression was very low. The His and Xpress antibodies each detected nonspecific proteins.

Aim 1.2

- ◆ After transient transfection of pcDCA4 ranBPM into HeLa cells, protein production *in vivo* was visualized by confocal microscopy. I could not make out distinct regions of ranBPM expression as demonstrated by Nakamura et al. I attribute this to the lack of specificity of the His and Xpress antibodies. We have generated a psoriasin specific antibody⁷ as well as two human breast cancer cell lines stably transfected with psoriasin and express it at a high level: the invasive cell line MDA-MB-231 (clone FD3) and the pre-invasive cell line MCF10AT3B (clone ZP1B3). RanBPM has been shown to co-localize to the centrosome in the HeLa epithelial cell line as well as in fibroblasts³. After proving that our cell lines expressed ranBPM by RT-PCR, we reasoned that ranBPM protein must be present in the centrosome. Using our psoriasin transfected cell lines as well as mock transfectants and the parent cells, I was able to co-localize psoriasin to the centrosome *in vivo* using an antibody to the centrosomal specific protein pericentrin (Fig 1).
- ◆ Because I could only get very low ranBPM expression by transient transfection, we decided switch our approach from immunoprecipitation of *in vivo* proteins to *in vitro* proteins. Using the pcDNA3.1 psoriasin construct with the T7 promoter, I used Sigma's TNT kit to produce *in vitro* ³⁵S-met labeled psoriasin. ³⁵S-met labeled ranBPM was generated the same way using the pcDNA4 HisMax construct. After mixing and incubating psoriasin and ranBPM labeled proteins together, I was able to show that they co-immunoprecipitate after electrophoresis through an acrylamide gel and autoradiography (Fig 2). This result proves the interaction seen originally in the Yeast 2-Hybrid Assay can be replicated by another technique.

TECHNICAL OBJECTIVE / AIM 2

1. 1-6 Construct ranBPM expression constructs for inducible stable-transfection in Tet ON MCF-7 cells.
2. 6-12 Stable transfection of MCF-7 cell line

Aim 2.1

- ♦ The entire protein coding cDNA sequence was cloned into the tetracycline inducible expression vector pTRE2 (Clontech) and had was confirmed by DNA sequencing.

Aim 2.2

- ♦ The pTRE2-ranBPM vector was linearized and co-transfected with the linearized background selection vector pTK-Hygro (Clontech) at a ratio of 1:10 respectively. The cell line used is MCF-7 that have been stably transfected so they express the r-tTa protein (Clontech) making these cells "Tet ON". Cells at various densities were transfected with varying amounts of transfection reagents and DNA. At 24 hours post transfection, the cells were placed on hygromycin selection (200µg/ml final concentration) and left for colony formation to develop over a 3-week period with media changes every 3-4 days. The experiment was repeated several times with no success. Transfection was tried with the background vector by itself and resulted in again no colonies. Others have advised me that the pTK-Hygro vector has failed in their hands as well depending on the cell type. We have since obtained the vector pTRE2-Hyg and have cloned ranBPM into it. This vector will eliminate the need for co-transfection, as well, the hygromycin selection gene is driven by the strongly active SV40 promoter. Efforts are currently underway to stably transfect this new construct into the MCF-7 "Tet ON" cells.

TECHNICAL OBJECTIVE / AIM 3

1. 1-6 Construct ranBPM in-situ hybridization probes for tissue analysis

Aim 3.1

- ♦ A 400 base pair region of ranBPM's cDNA has been cloned into the pGEM-T vector. This will allow the production of sense RNA probe from the T7 promoter and anti-sense RNA probe from the SP6 promoter.

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstrate psoriasin protein co-localizes to the centrosome in two different human breast cancer cell lines using confocal microscopy allowing the psoriasin/ranBPM interaction to occur *invivo* in human cells
2. Show the psoriasin/ranBPM protein interaction by co-immunoprecipitation to confirm the original observation in the Yeast 2-Hybrid Assay

REPORTABLE OUTCOMES

Abstracts

Ethan D. Emberley, A. Kate Hole, R. Daniel Gietz, Leigh C. Murphy and Peter H. Watson. INTERACTION OF THE DIFFERENTIALLY EXPRESSED S100A7 GENE WITH CENTROSOMAL PROTEINS. San Antonio Breast Cancer Symposium. December 2000.

Ethan D. Emberley, A. Kate Hole, R. Daniel Gietz, Leigh C. Murphy and Peter H. Watson. A DIFFERENTIALLY EXPRESSED BREAST CANCER GENE AND ITS INTERACTION WITH CENTROSOMAL PROTEINS. University of Manitoba Research Day. June 2001.

Oral Presentation

Ethan D. Emberley, R. Daniel Gietz, Leigh C. Murphy and Peter H. Watson. THE DIFFERENTIALLY EXPRESSED PSORIASIN GENE: POTENTIAL ROLE IN BREAST CANCER PROGRESSION. Canadian Breast Cancer Research Initiative, Reasons for Hope. May 2001

CONCLUCIONS

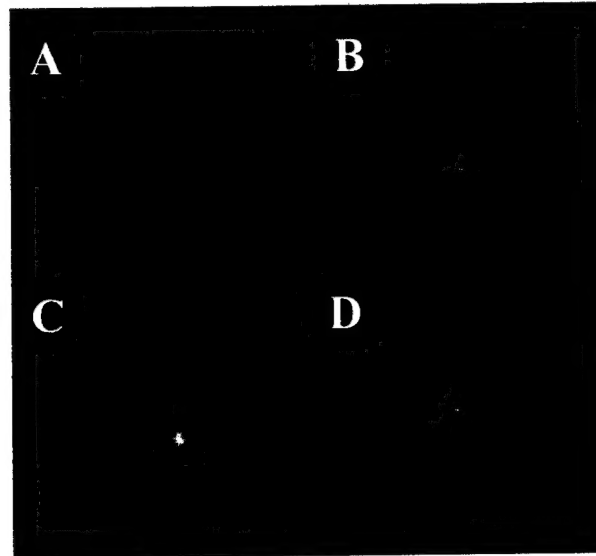
The observation of psoriasin's differential expression pattern in breast cancer makes it a candidate "invasion gene". By attempting to identify a biological function for this gene in the complex and poorly understood process of invasion, we would be one step closer to understanding what makes a pre-invasive cell become invasive. This has relevance in terms of patient treatment and prognosis as well as being a future target for drug therapy.

References

- 1 Ernster VL, Barclay J, Kerlikowske K, Grady D, Henderson C. Incidence of and treatment for ductal carcinoma in situ of the breast. *JAMA* 1996 Mar 27;275(12):913-8
- 2 Leygue E, Snell L, Hiller T, Dotzlaw H, Hole K, Murphy LC, Watson PH. Differential expression of psoriasin messenger RNA between in situ and invasive human breast carcinoma. *Cancer Res* 1996 Oct 15;56(20):4606
- 3 Nakamura M, Masuda H, Horii J, Kuma Ki, Yokoyama N, Ohba T, Nishitani H, Miyata T, Tanaka M, Nishimoto T. When overexpressed, a novel centrosomal protein, RanBPM, causes ectopic microtubule nucleation similar to gamma-tubulin. *J Cell Biol* 1998 Nov 16;143(4):1041-52
- 4 Duensing S, Munger K. Centrosome abnormalities, genomic instability and carcinogenic progression. *Biochim Biophys Acta* 2001;1471(2):M81-8
- 5 Lingle WL, Salisbury JL. The role of the centrosome in the development of malignant tumors. *Curr Top Dev Biol* 2000;49:313-29
- 6 Salisbury JL, Whitehead CM, Lingle WL, Barrett SL. Centrosomes and cancer. *Biol Cell* 1999 Jul;91(6):451-60
- 7 Al-Haddad S, Zhang Z, Leygue E, Snell L, Huang A, Niu Y, Hiller-Hitchcock T, Hole K, Murphy LC, Watson PH. Psoriasin (S100A7) expression and invasive breast cancer. *Am J Pathol* 1999 Dec;155(6):2057-66

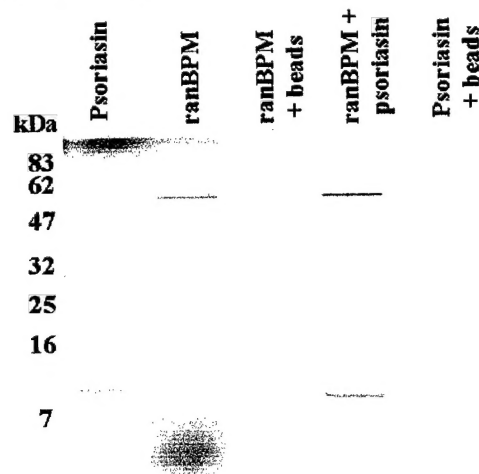
APPENDICES

Figure 1: Co-localization of psoriasin and pericentrin in human breast cells



Confocal Immunofluorescence showing cellular localization of (panel A) psoriasin (FITC), and (panel B) pericentrin (Cy5) in MDA-MB-231 cells stably transfected with psoriasin expression vector (clone FA1). Panel (C) shows overlay of images (A) and (B). Panel (D) is the equivalent of panel (C) with a control MDA-MB-231 cell line (VA1) that is stably transfected with the empty expression vector showing only pericentrin signal.

Figure 2: Co-immunoprecipitation of psoriasin with RanBPM



Autoradiography of Western Blot showing radiolabeled psoriasin protein (first lane from left) and ranBPM protein (second lane from left) alone. Middle lane indicates that an antibody to ranBPM is needed in order to immunoprecipitate it. Lane second from right shows psoriasin protein interacting with ranBPM protein (immunoprecipitation). Far right lane demonstrates the specificity for that antibody to ranBPM and not psoriasin.